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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF PREVENTION, PESTICIDES AND TOXIC SUBSTANCES

MAR 19 2009

CONTAINS FIFRA CONFIDENTIAL BUSINESS INFORMATION

MEMORANDUM

SUBJECT: C5 Honeysweet Plum containing the coat protein gene of plum pox virus.

FROM: Joel V. Gagliardi, Ph.D., Microbial Ecologist
Microbial Pesticides Branch, Biopesticides and Pollution Prevention Division (7511P)

Joel V. Gagliardi

TO: Denise Greenway, M.S., Regulatory Action Leader
Microbial Pesticides Branch, Biopesticides and Pollution Prevention Division (7511P)

THROUGH: John L. Kough, Ph.D., Senior Scientist
Microbial Pesticides Branch, Biopesticides and Pollution Prevention Division (7511P)

John L. Kough

ACTION REQUESTED:
Review submitted registration and food tolerance exemption related materials for registration of C5 Honeysweet Plum resistant to the Plum Pox Potyvirus.

CONCLUSION:
Product Characterization; Allergenicity-Homology Studies – **ACCEPTABLE**; Oral toxicology and In-Vitro Digestibility waiver requests **ACCEPTABLE**. Food Tolerance Exemption Petition – **ACCEPTABLE** – C5 Plum Pox Viral Coat Protein in or on Fruit, Stone, Group 12 and Almond.

DATA REVIEW RECORD

Active Ingredient:	Coat protein gene of plum pox virus.
Product Names:	C5 Honeysweet Plum.
Company Name:	U.S. Department of Agriculture, Agricultural Research Service.
EPA Reg. Nos.:	11312-R, 11312-I.
Chemical Number:	006354.
Decision Numbers:	380391, 380394.
DP Barcodes:	342996, 343005, 343006, 347035, 355577, 355580.
MRID Nos.:	471573-01; 471573-02; 471573-03; 471749-02; 474749-03.

BACKGROUND:

C5 Honeysweet plum pox virus (PPV) resistant trees are intended for nursery or orchard use wherever PPV is or may become endemic. According to USDA-APHIS, PPV was first described in 1915 in Europe and by 1999 had entered the U.S. via a Pennsylvania orchard. Current distribution of PPV along the Canadian border and recent outbreaks in New York and Michigan underscore that this virus is becoming endemic despite efforts to contain outbreaks by bulldozing and disposing of infected vegetation, moratoriums on movement of any materials containing these plant pests, accompanied by vector control. The nature of the resistance gene is such that only viruses similar to the originating strain of plum pox virus are expected to be affected by the complementary RNA fragments that ultimately cause the natural plant protection mechanism, post-transcriptional gene silencing (PTGS), to resist further virus infection. While devastating to agriculture, plum pox virus (PPV) is primarily an economic pest since the primary effect is to reduce fruit quality and in some cases fruit yield, upon infection. Other stone fruit (*Prunus*) that are affected by PPV strains and therefore may serve as source and sink for PPV include peaches, other plums, almonds, nectarines, both sweet and sour cherries [http://www.apsnet.org/online/feature/PlumPox/plumpoxfs.pdf], native or wild *Prunus* species, and additionally various other (non *Prunus*) dicotyledonous plants [http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/00.057.0.01.054.htm].

REVIEW SUMMARY:

Study Type: Product Identity (OPPTS 885.1100); Manufacturing Process (OPPTS 885.1200); Discussion of Formation of Unintentional Ingredients (OPPTS 885.1300); Analysis of Samples (OPPTS 885.1400); Certification of Limits (OPPTS 885.1500); Physical and Chemical Characteristics (OPPTS 830.6302-830.7950).

MRID Nos.: 471573-01; 474749-03.

Test Material: C5 HoneySweet Plum – Resistant to Plum Pox Virus (Plum Pox Viral Coat Protein Gene).

Study Summary: All aspects of the development process for C5 HoneySweet Plum trees expressing the Plum Pox Virus Resistance Gene (from a plant-infecting Potyvirus) have been previously published as peer-reviewed manuscripts. The cloning vehicle used to express the Plum Pox Viral Coat Protein Gene is well described as to bacterial and plant promoters and genes expressing antibiotic resistance and reporter gene phentotypes for use in selection and differentiation of transformed versus untransformed cells. Inserts into plum from *Agrobacterium*-mediated transformation were characterized by PCR, ELISA, sequencing and other relevant techniques and are also publically available as submitted to the USDA-APHIS-BRS in their application [http://www.aphis.usda.gov/brs/aphisdocs/04_26401p.pdf]. One complete plasmid insert (lacking bacterial expressed areas) is evident, as are four other partial inserts. One of the partial inserts is an inverted repeat of the coat protein that is unlikely to be translated. This particular event, C5, was selected due to the target inserts, lack of antibiotic resistance genes under bacterial promoters, and ability to resist Plum Pox Virus. Under field conditions, C5 trees remained symptom free despite exposure from grafting activities and by natural aphid vector exposure, where other, susceptible trees clearly were all detrimentally affected by the virus progressively over several years. The postulated mode of action is RNA production from the opposite strand carrying the inverted repeat which can combine to form double stranded RNA with mRNA from the intact coat protein gene insert. Post-transcriptional gene silencing (PTGS) in the plant then acts to recognize similar sequences in ~23 base pair increments, leading to quick degradation of matching RNA, methylation of the corresponding DNA gene and subsequently down-regulation of mRNA production from the coat protein gene

on the chromosome. The same plant activity works to degrade any infecting viral RNA, preventing viral replication and further infection. Since the mechanism of resistance is a natural part of the plant, and the active ingredient is identical to the infecting RNA virus or its replication intermediate, this is a very low-risk approach to virus resistance. Essentially the C5 plum just displays an earlier plant response to what happens if the virus infects a non-engineered plum, allowing for destruction of the viral genome before plant effects, such as chlorosis or degradation of plum fruits, can develop.
Classification: ACCEPTABLE.

Study Type: Waiver request for: Acute Oral Toxicity/Pathogenicity (OPPTS 152-30 / 885.3050); Acute Toxicology, Tier II (OPPTS 885.3550); Subchronic Toxicity/Pathogenicity (OPPTS 885.3600); Chronic Feeding Study, Tier III (OPPTS 152-50).
MRID Nos.: 471573-03; 494749-02.
Test Material: Plum Pox Viral Coat Protein.
Study Summary: There are several pertinent issues for discussion concerning potential for dietary hazards from C5 Honeysweet Plum. The first is whether there are any novel exposures to proteins that may be toxic, allergenic or that may have antinutrient qualities. Sufficient information has been reviewed in general that indicates that naturally occurring proteins of plant viruses, including the coat protein from Plum Pox Potyvirus, do not possess any qualities that may lead to toxicity, allergenicity or action as an antinutrient when ingested. Bolstering this assessment is a recent study that looked at RNA virus prevalence in the human gastrointestinal tract and found "[t]he vast majority of the 36,769 viral sequences obtained were similar to plant pathogenic RNA viruses. The most abundant fecal virus in this study was pepper mild mottle virus (PMMV), which was found in high concentrations—up to 10^9 virions per gram of dry weight fecal matter". Another key point is the Potyviridae contain one or more polyproteins that are produced directly from virus mRNA, which then self-cleaves into individual proteins, including the coat protein. Exposure in foods from a natural plant virus infection therefore is to a wider range of proteins than just the coat protein. Also pertinent is the mode of resistance elicited by transcription of DNA antisense to the mRNA of the Plum Pox Virus coat protein. Post-transcriptional gene silencing virtually eliminates the possibility of translation of protein from mRNA since homologous mRNA (plant- or virus-expressed) is quickly cleaved. Therefore, exposure to any proteins from the Plum Pox Virus will be virtually eliminated since the mRNA from infecting virus would not be able to replicate under foreseeable agronomic circumstances. Also pertinent to this discussion is the product of the plum pox virus coat protein gene as inserted into the C5 Honeysweet Plum. In the original virus and its replication intermediates, DNA is not required since a virus-encoded RNA-Dependent RNA-polymerase is used. To express the gene in a plant, a DNA copy must be incorporated so that the plant will express mRNA homologous to the virus coat protein only. Often for a Potyvirus this means also adding a start codon and short leader sequence since the viral start codon is distant from the coat protein sequence in the normal viral RNA genome. As mentioned in this petition a full sequence analysis and database search was undertaken including the putative protein sequence of the Plum Pox Virus coat protein gene inserted into C5 Honeysweet Plum. These results (reviewed in the next sections) indicate no similarity to known toxins, allergens or antinutrients. Considering potential production of a protein even when no measurable protein is found is important since the silenced inserted gene has the capability to produce a protein (has an open reading frame) and in some cases PTGS can be suppressed. Known instances where suppression of gene-silencing can occur include low temperature growth and production of PTGS inhibitors. There are however, no foreseeable events that would cause a breakdown in resistance for C5 HoneySweet plum to Plum Pox Virus in the field.
Classification: ACCEPTABLE.

Study Type: Allergenicity-Homology Study; Report of Hypersensitivity Incidents; In-Vitro Digestibility Study Waiver.
MRID Nos.: 471573-03; 474749-02; 474749-03.
Test Material: Transgene and overlapping sequences of C5 HoneySweet Plum.
Study Summary: Clones of the inserts (or the putative coat protein) were chosen for analysis due to the possibility for sequence overlap and protein production other than from transgene open reading frames, and the need to assess the actual insert not the intended clone. Open-reading frames analysis was used to compare any alignments of potential allergens and antinutrients to further narrow down any potential hazard. None of the sequence analyses produced alignments of >35% and in the few cases where a direct amino acid match was found, they were common sequences and below the hazard threshold of an 8 amino acid homology currently utilized as a benchmark. Therefore, no hazard potential for allergenic or antinutrient capability apparently exists from the transgenes and overlapping plum DNA expression in the C5 HoneySweet plum. No further testing, including an in-vitro digestibility assay, is required. The registrant also included a statement that since experimentation began with C5 HoneySweet plum in 1992 approximately 80 trees have been tested. ARS production staff numbering approximately 20 people in the U.S. (West Virginia) and personnel performing testing in Spain, Poland, Romania, the Czech Republic and Chile, have not experienced known hypersensitive or other adverse effects.
Classification: ACCEPTABLE.

Study Type: Tolerance Exemption Petition: Plum Pox Viral Coat Protein in or on Fruit, Stone, Group 12 and Almond.
MRID Nos.: None.
Test Material: C5 HoneySweet Plum – Resistant to Plum Pox Virus (Plum Pox Viral Coat Protein Gene).
Study Summary: The submitted data and gathered background information on the safety of plant virus proteins, including coat proteins in foods and namely the C5 clone that can express the Plum Pox Virus coat protein with short leader sequence from C5 HoneySweet Plum, led to the conclusion that the C5 Plum Pox Virus Resistance is low risk and safe for consumption in foods. Due to outcrossing potential and the various uses of rootstocks and grafts, a wider tolerance exemption petition encompassing all stone fruits was reviewed. The safe for consumption and low risk finding holds equally for all foods in this group. In addition to a long history of consumption without known toxic, allergenic or antinutrient effects for all proteins from plant viruses, the C5 specific sequence and putative coat protein representing genome sequencing comprising ~80% of the transgenes was used to search databases for any match. No qualifying toxic, allergenic or antinutrient sequence alignments were found. In the specific case of C5 Honeysweet Plum the mode of resistance is post-transcriptional-gene-silencing, which renders the C5 genome incapable of producing mRNA to express protein under normal agronomic circumstances. No PPV coat protein has been detected to date in these plum trees or fruits. In addition, infecting Plum Pox Virus is prevented from expressing symptoms and in many cases infection is prevented, thereby lowering exposure to protein even from the naturally occurring virus. While the safe for consumption and low-risk findings here were not based on the premise that exposure cannot occur, actual exposure to Plum Pox Virus proteins and to the cloned PPV coat protein is expected to be much lower (possibly non-existent) compared to PPV-infected trees and fruits.
Classification: ACCEPTABLE – C5 Plum Pox Viral Coat Protein in or on Fruit, Stone, Group 12 and Almond.

***** CONTAINS FIFRA CONFIDENTIAL BUSINESS INFORMATION *****

DATA EVALUATION RECORD

Review by: Joel V. Gagliardi, Ph.D.
Secondary Review by: John L. Kough, Ph.D.

Study Type	Product Identity (OPPTS 885.1100); Manufacturing Process (OPPTS 885.1200); Discussion of Formation of Unintentional Ingredients (OPPTS 885.1300); Analysis of Samples (OPPTS 885.1400); Certification of Limits (OPPTS 885.1500); Physical and Chemical Characteristics (OPPTS 830.6302-830.7950).
MRID No.	471573-01; 474749-03.
Test Material	C5 HoneySweet Plum – Resistant to Plum Pox Virus (Plum Pox Viral Coat Protein Gene).
Study Nos.	IR-4 PR No. 0377B
Sponsor	Ralph Scorza, Ph.D.; USDA-ARS, Appalachian Fruit Research Station; Kearneysville, West Virginia.
Testing Facility	USDA-ARS, Appalachian Fruit Research Station; Kearneysville, West Virginia.
Titles of Reports	Group A – Product Analysis Test Guidelines; Responses to Questions Concerning Plum Pox Virus Resistant HoneySweet Plum.
Authors	Michael Braverman, Ph.D.; Ralph Scorza, Ph.D.
Studies Completed	June 1, 2007; July 10, 2008.
Study Summary	All aspects of the development process for C5 HoneySweet Plum trees expressing the Plum Pox Virus Resistance Gene (from a plant-infecting Potyvirus) have been previously published as peer-reviewed manuscripts. The cloning vehicle used to express the Plum Pox Viral Coat Protein Gene is well described as to bacterial and plant promoters and genes expressing antibiotic resistance and reporter gene phentotypes for use in selection and differentiation of transformed versus untransformed cells. Inserts into plum from *Agrobacterium*-mediated transformation were characterized by PCR, ELISA, sequencing and other relevant techniques and are also publically available as submitted to the USDA-APHIS-BRS in their application [http://www.aphis.usda.gov/brs/aphisdocs/04_26401p.pdf]. One complete plasmid insert (lacking bacterial expressed areas) is evident, as are four other partial inserts. One of the partial inserts is an inverted repeat of the coat protein that is unlikely to be translated. This particular event, C5, was selected due to the target inserts, lack of antibiotic resistance genes under bacterial promoters, and ability to resist Plum Pox Virus. Under field conditions, C5 trees remained symptom free despite exposure from grafting activities and by natural aphid vector exposure, where other, susceptible trees clearly were all detrimentally affected by the virus progressively over several years. The postulated mode of action is RNA production from the opposite strand carrying the inverted repeat which can combine to form double stranded RNA with mRNA from the intact coat protein gene insert. Post-transcriptional gene silencing (PTGS) in the plant then acts to recognize similar sequences in ~23 base pair increments, leading to quick degradation of matching RNA, methylation of the corresponding DNA gene and subsequently down-regulation of mRNA production from the coat protein gene on the chromosome. The same plant activity works to degrade any infecting viral RNA, preventing viral replication and further infection. Since the mechanism of resistance is a natural part of the plant, and the active ingredient is identical to the infecting RNA virus or its replication intermediate, this is a very low-risk approach to virus resistance. Essentially the C5 plum just displays an earlier plant response to what happens if the virus infects a non-engineered plum, allowing for destruction of the viral genome before plant effects, such as chlorosis or degradation of plum fruits, can develop.
Classification	**ACCEPTABLE.**
Good Laboratory Practice	The research was not conducted in compliance with 40CFR Part 160, but is considered scientifically valid.

Test Material: C5 HoneySweet Plum – Resistant to Plum Pox Virus (Plum Pox Viral Coat Protein Gene).

I. PRODUCT IDENTITY AND COMPOSITION: Slices of the hypocotyl segment from fresh (or recently stored at 4°C) plum (*Prunus domestica*) cultivar 'BlueByrd' seeds are removed under sterile conditions for transformation. Approximately 8% of slices incubated on selective-differential growth

media (50 µg/mL Kanamycin and GUS at 37°C) were able to produce shoots and roots following *Agrobacterium*-mediated transformation with binary plasmid pGA482GG/PPV-CP-33. The cloned plum pox virus coat protein was under the PPV-CP-33 plasmid-carried CaMV 35s promoter subcloned into the pGA482GG plasmid HindIII site after restriction enzyme digestion, then transformed to *Agrobacterium tumefaciens* strain C58.Z707 and grown in selective media using kanamycin (50 µg/mL) and gentamicin (50 µg/mL) and tested for GUS activity with an X-Glu solution. Presumptive transformants containing the desired cassette (Fig. 1 from page 27, MRID 471573-01 *below*) were confirmed by multiplex PCR for the PPV-CP, nptII and GUS genes as inserted. In all there are 5 mapped inserts into the selected plum event (C5) including one complete and four partial inserts. There is one complete, and one doubled (tail-to-tail) PPV-CP insert (Fig. 6 from page 37, MRID 471573-01 *below*). Each PPV-CP insert has a leading 35s CaMV promoter and short untranslated region from the TMV virus in the polycloning site present (see Fig. 6 from page 37 of 89, MRID 471573-01 *below*). Potentially this would produce 3 copies of mRNA for the PPV-CP from the PPV-D strain with an added ATG start codon and TMV leader sequence. Stability was assessed repeatedly from 1990-2005 in propagated transformants and progeny to confirm retention of the inserts, all of which appear to be linked but are an unknown distance from each other on the chromosome.

RNA transcripts were characterized using blots, aimed at the expected 1.4 kb PPV-CP target. Transformants C2, C3 and C4 produced detectible transcripts within 5 hours with blot exposure (32-P hybridization). C5 transcript was barely detected after 40 hours when compared to untransformed controls. No transcript was detected for C6 and no coat protein production was found in either the C5 or C6 events. Analysis of inserts by restriction enzyme digestion and DNA gel blot showed the expected internal PPV-CP BamHI 1.2 kb fragment plus an approximately double-sized fragment from C5. Several EcoRI digest bands besides the expected 7 kb band further indicated multiple, and different, insertions compared to the pGA482GG/PPV-CP-33 plasmid control. In C5 EcoRI bands at 1.9, 3, 5, 7 and 10 kb hybridized with PPV-CP; the 5 and 10 kb bands also hybridize with a probe for nptII and the 7 kb band with uidA. Another 20 kb fragment only hybridized with nptII.

Sequencing of the inserts was accomplished but only about 80% determined "due to sequence repeats, DNA methylation, and the presence of an origin of replication in the insert". The proposed structures (see Fig. 6 from page 37 of 89, MRID 471573-01 *below*) as discussed above are in five parts, one of them complete the others containing duplications or rearrangements and one is listed as an inverted repeat of the PPV coat protein. The mechanism for inversion is not stated though this insert "may be critical for providing PPV resistance".

A. <u>INERT INGREDIENT GENES UTILIZED IN DEVELOPMENT</u>:

An ampicillin resistance gene (β-lactamase) is present as part of the construct from plasmid pBR322 fragments engineered into plasmid pGA482GG-PPV-CP. However the gene has a bacterial insert containing a *cos* site (from cosmid MUA10 as derived from pBR322), and is inactivated. Non-functionality of this gene was demonstrated by RNA extraction, and reverse-transcriptase PCR using a reverse primer and spanning the bacterial insert. Analysis of the original plasmid construct but in *E. coli* DH5-α using gentamicin to select for the plasmid, and control *E. coli* DH5-α grown in broth but without the plasmid, were plated to LB agar containing 100 mg/L ampicillin. This experiment was replicated twice. Each trial resulted in five colonies of *E. coli* DH5-α containing plasmid pGA482GG-PPV-CP that reverted to functional β-lactamase by deletion of the *cos* insert. No spontaneous ampicillin-resistant colonies resulted from *E. coli* DH5-α without the plasmid. Ten C5 plum leaf samples taken from 1997-2005 were assayed for β-lactamase mRNA using reverse primers specific to a 532 bp region spanning the *cos* site. No mRNA was detected from the samples (archived at -80 °C) taken at various months throughout those years. Positive controls were the uidA sequence for the GUS transgene, and CAB for the plant chlorophyll A/B – both produced positive

PCR results in all 10 samples. Supplemental information provided during APHIS review confirmed by PCR that the *cos* site remained in the C5 inserted gene. It is evident that even in the highly selective environment of fast-growing *E. coli* cells in presence of ampicillin and harboring a high-copy-number plasmid, that the mutation rate to active amp-R is exceedingly low. None of these conditions is expected to occur for C5 HoneySweet plum trees or fruit.

Other selective marker antibiotic genes present on the original plasmid were tetracycline and gentamycin resistances, present on the part of the plasmid under a bacterial promoter. EPA requested that the registrant provide proof that these genes were absent in the C5 plum trees. On July 10, 2008 the registrant supplied new laboratory tests on extracts of tree fruit and leaves, both the wild-type (BlueByrd) and the PIP (C5). The positive control, plasmid pGA482GG-PPV-CP response is clear, while all lanes of test sample PCR product is negative for the tetracycline and gentamycin resistance genes. PPV-CP DNA is detected from the plasmid and C5 leaves but not from C5 fruit (MRID 474749-02). All genes were absent from the wild-type BlueByrd fruit and leaf samples. The registrant reports that simultaneous tests for nptII, PPV-CP, uidA and 26s rDNA produced requisite PCR products though these results were not shown on these gel photos (page 8, MRID 474749-03). The gene for nptII (neomycin phosphotrasnferase II, or kanamycin resistance) is present and does produce both mRNA and protein from the inserted *Agrobacterium tumefaciens* NOS promoter. There are four copies of the nptII gene inserted into the C5 chromosome, of which at least three are thought functional. There is a tolerance exemption for neomycin phosphotransferase II at 40CFR174.521. Another inert is the protein from the uidA gene, GUS (*E. coli* β-D-glucuronidase), under direction of a CaMV 35s promoter. There are 2 complete copies of the uidA gene on separate inserts, and three fragments on two of the other inserts. GUS has an existing tolerance exemption at 40CFR174.525.

B. <u>ACTIVE INGREDIENT DESCRIPTION</u>:

The active ingredient as inserted is a reverse transcription derivation of the virus coat protein RNA, inserted with a 3' untranslated region with fusion of a start codon and short leader sequence and an *Agrobacterium tumefaciens* NOS terminator under direction of a CaMV 35s promoter. There is one complete copy of the PPV-CP gene, a small fragment of the PPV-CP 35s promoter on another insert and a third insert that is a "3'-3' tail-to-tail copy of the PPV-CP with the 35S promoter for each copy and a portion of GUS sequence flanking each PPV-CP copy. The insert [is] flanked by plum DNA."

C. <u>PPV RESISTANCE AND MODE OF ACTION</u>:

The reviewer postulates that coat protein mRNA is transcribed as engineered from the single whole PPV-CP insert, and simultaneously, mRNA from the complementary DNA strand of the inverted-repeat 3'-3' insert is transcribed via a plant open reading frame, leading to double-stranded RNA for the PPV-CP sequence. It is this likely mode of action that provides the described plum pox virus resistance through the plant Post Transcriptional Gene Silencing (PTGS) system. C5 plum was shown resistant to the four major serotypes of plum pox virus (Ravelonandro et al. 2001) by a method other than PPV coat protein production in the plant. Subsequent work narrowed the mechanism of resistance to PTGS noting there were low mRNA levels and high methylation of PPV-CP DNA sequences relating to resistance, upon challenge with PPV (Scorza et al. 2001). C5 trees were selected from testing among five transgenic plums from the same event - C2, C3, C4, C5, C6 - (Scorza et al. 1994) all confirmed by multiplex PCR for the PPV-CP, nptII and GUS genes as inserted (see above). Field experiments in Poland with replicates of each transgenic plum and a wild-type, were chip-bud inoculated in 2 out of 10 replicates, then exposed to natural aphid populations over two years (2003, 2004); as a result, non-inoculated C5 trees were the only

ones not infected in the field (Hily et al. 2004). Chip-bud inoculated C5 replicates showed mild symptoms along the chip-bud branches with symptoms decreasing over several more years. Though symptoms had abated, ELISA and IC-RT-PCR testing showed presence of the *Plum pox virus* in a few samples of C5, mainly those still showing symptoms of infection. The other susceptible transgenic plums and the wild-type (highly susceptible B70146) all developed progressively worsening symptoms. B70146 had readily visible chlorotic symptoms within 1-2 years. Another field trial, in Spain, tried variations of non-transgenic wild-type plum and fewer of the transgenic plums tested in Poland. Results were confirmatory that C5 had durable resistance to inoculated or field acquired wild-type *Plum pox virus*. Even when infected through rootstock, C5 trees were able to check spread and symptoms of the virus to near the site of the graft. Further testing of hybrids and seedlings showed that "the multiple transgene inserts of C5 are closely linked and are transmitted as a single dominant gene (locus)" (Scorza et al. 1998) though PTGS-based resistance in germinated seedlings may be delayed up to a month. In MRID 474749-02 the inverted repeat of the PPV-CP gene was further clarified: a piece of the 35s PPV-CP promoter is present on either end followed by a portion of the PPV-CP DNA without the NOS terminator. It is unknown if the sense strand of this insert can produce mRNA or protein, though as discussed above the antisense strand may produce mRNA from a plant open reading frame.

Hily, J.M., R. Scorza, T. Malinowski, B. Zawadzka and M. Ravelonandro. 2004. Stability of Gene Silencing-Based Resistance to *Plum pox virus* in Transgenic Plum (*Prunus domestica* L.) under field conditions. Transgenic Res. 13: 427-436.

Ravelonandro, M., P. Briard and R. Scorza. 2001. Significant Resistance of Transgenic Plums Against the Four Serotypes of Plum Pox Potyvirus. Proc. 18th Int. Symp. On Fruit Tree Virus Diseases (M.F. Clark *ed.*), Acta Hort. 550, ISHS 2001.

Scorza, R. M. Ravelonandro, A.M. Callhan, J. Cordts, M. Fuchs, J. Dunez and D. Gonsalves. 1994. Transgenic plums (*Prunus domestica* L.) express the plum pox virus coat protein gene. Plant Cell Reports 14: 18-22.

Scorza, R., A.M. Callahan, L. Levy, V. Damsteegt and M. Ravelonandro. 1998. Transferring Potyvirus Coat Protein Genes through Hybridization of Transgenic Plants to Product *Plum pox virus* Resistant plums (*Prunus domestica* L.). Acta Hort 472: 421-427.

Scorza, R., A. Callahan, L. Levy, V. Damsteegt, K. Webb and M. Ravelonandro. 2001. Post-Transcriptional Gene Silencing in Plum Pox Virus Resistant Transgenic European Plum Containing the Plum Pox Potyvirus Coat Protein Gene. Transgenic Research 10: 201-209.

II. DESCRIPTION OF STARTING MATERIALS AND PRODUCTION PROCESS:

The parent tree, *Prunus domestica* L. var. *domestica* (European plum) is found or cultivated in 13 continental states in the U.S. and was introduced from Europe (www.plants.usda.gov). European plum is not listed as a noxious weed and these trees are not commonly considered invasive or difficult to control in the U.S. Field trials conducted in PPV endemic regions of Poland, Romania and Spain showed that none of the C5 trees acquired PPV infection from natural aphid inoculation. Using only the coat protein gene of plum pox virus eliminates the possibility of producing an infectious agent from the transgene alone. The genes for selectable markers (nptII and GUS) used in tissue culture would not affect the survival of trees in the environment since their enzymatic substrates are not commonly found there. The mode of action of the active ingredient is to catalyze a common plant defense mechanism, called post-transcriptional gene silencing (PTGS) that is highly specific to the plum pox virus coat protein gene. Successful gene flow in the environment, given the inefficiency of even artificial crosses, is considered highly unlikely. A brief summary of provided references dating from the 19th-21st centuries on breeding in *Prunus* follows:

i. Natural incompatibility of hexaploid *P. domestica* with other *Prunus* species, most of them diploid;
ii. Very low percentages of fruit set from artificial or "forced" hybridization;
iii. Tendency of hybrids to be produced using *P. domestica* as a female parent rather than as a male or pollen parent;
iv. The extremely low hybridization rate with self-incompatible *P. domestica* ('Honeysweet' being self-incompatible);
v. The low vigor of hybrid seedlings; and
vi. The low fertility of hybrids that do survive.

Specifically, in the unlikely event a viable cross is made with *P. domestica* [0% most often but artificial crosses with success up to 1.3% are reported] they are often not vigorous or fertile, and the only known cross in cultivation, 'Alhambra' is three generations removed from crossing with *P. domestica*. While data on ability to cross with indigenous plum is not readily available or is incomplete, the genome incompatibility (mainly diploid for natives, hexaploid for C5 Honeysweet), the low fertility of any successful crosses, and the very low frequency of even artificial crosses make this a very small probability. Using C5 plum trees as rootstock for *Prunus spinosa* (Ravelonandro et al. 2001) would not result in any known transgene flow to the grafted tree, though PTGS could transfer into *P. spinosa* to provide PPV resistance.

Ravelonandro, M., P. Briard, R. Renaud and R. Scorza. 2001. Transgene-Based Resistance to Plum Pox Virus (Sharka Disease) Transferred Through Interspecific Hybridization in *Prunus*. Proc. Int. Symp. On Molecular Markers (Dore, Dosba and Baril, *Eds.*). Acta Hort. 546, ISHS 2001.

Deficiencies: None.

III. DISCUSSION OF FORMATION OF IMPURITIES: Experimentation has demonstrated stability of the transgenes and tendency to transfer as a single dominant gene locus during breeding, so formation of impurities is unlikely in C5 plum trees.

Deficiences: None.

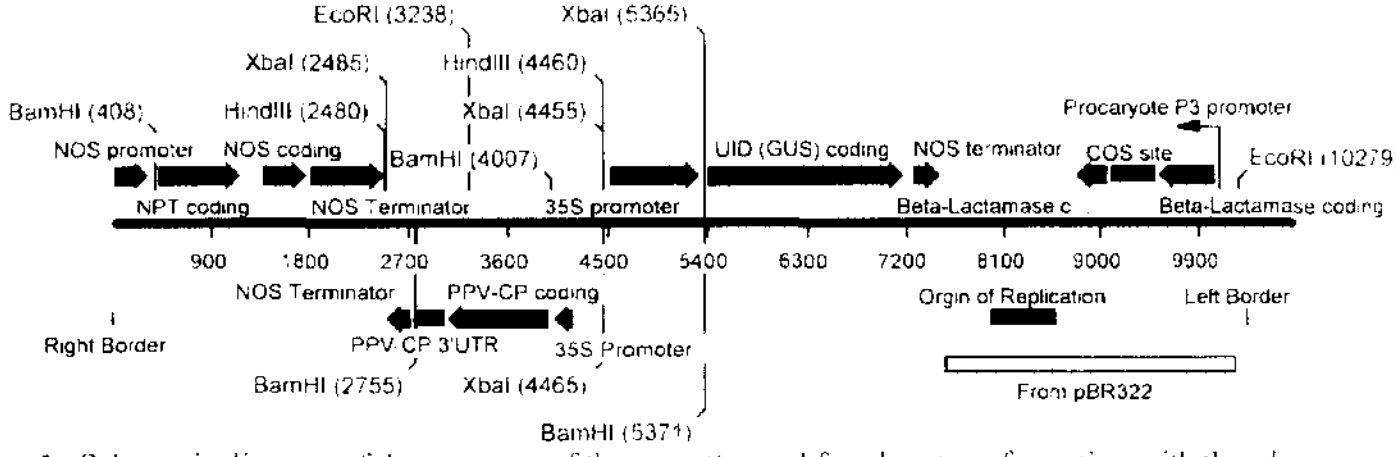

Figure 1. Schematic diagram of the structure of the cassette used for plum transformation with the *plum pox virus* (PPV) coat protein (CP) gene. Blue indicates coding regions, green = promoters, red = terminators. The portion of the insert derived as a cloning artifact from plasmid pBR322 is indicated with a light green bar with the origin of replication indicated in red. The *Bam*HI-isolated PPV-CP fragment is 1.2 kb.

From: page 27, MRID 471573-01.

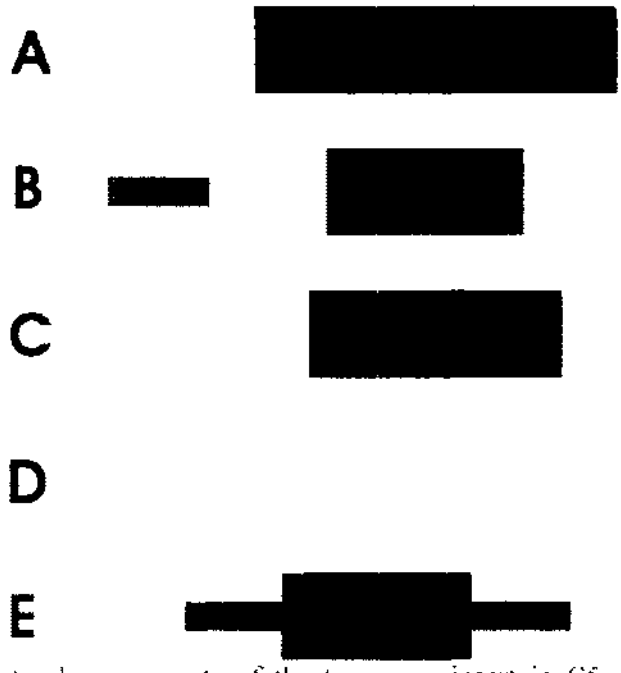

Figure 6. Proposed structural components of the transgene insert in C5 as determined by DNA blot analyses and sequencing. Yellow NPTII, red PPV-CP, blue GUS, green sequence from pBR322, purple plum DNA sequences. Genetic analyses have suggested that these insert pieces, A through E, are linked. The precise distance, one from another is not known.

C5 contains the following transgene fragments as illustrated in **Fig. 6**:

A. A complete insert consisting of the NPT, PPV-CP and GUS genes and their respective promoters plus sequence originating from pBR322 (see **Fig. 1**).
B. A fragment consisting of the NPTII gene with the nopaline synthase (nos) promoter and part of the GUS gene, the pBR322 segment and another copy of the NPTII gene with nos promoter, bordered at the 5' position by plum DNA.
C. The NPTII gene, the GUS gene with a small segment of the 35S promoter from the PPV-CP gene, and part of the pBR322 segment.
D. A single copy of the NPTII gene (it is not known if the nos promoter is present).
E. A 3'-3' tail-to-tail copy of the PPV-CP with the 35S promoter for each copy and a portion of GUS sequence flanking each PPV-CP copy. The insert flanked by plum DNA.

From: page 37, MRID 471573-01.

V. CERTIFIED LIMITS:

TABLE 4. Nominal CSF concentrations and certified limits for

Ingredients (CAS number)	PC Code	Purpose	Concentration (% by weight)		
			Nominal	Lower	Upper
Active Ingredient					
Plum Pox Virus Resistance Gene (Plum Pox Viral Coat Protein Gene) DNA	006354	Active ingredient	0.0005	0.00045	0.00055
Inert Ingredients					
Neomycin phosphotrasnferase nptII * CAS number 62213-36-9	806304	Selective marker / Inert ingredient	0.0014	0.0009	0.0015
Beta-Glucuronidase GUS * CAS number 9001-45-0	829082	Differential marker / Inert ingredient	0.0010	0.0009	0.0011
Other native DNA and plant material	None	Inert ingredient	99.997	96.997	102.9997

[a] Data from CSF dated 6/13/2008.

Deficiencies: None.

VI. ENFORCEMENT ANALYTICAL METHOD: As provided in MRID 474749-02 the C5 transformation event of BlueByrd may be differentiated from untransformed trees using the following PCR primers for the inserted coat protein gene:

PPV-CP (1 kbp)

Forward: 5'-AAGCTGACGAAAGAGAGGACGAG-3'

Reverse: 5'-CTACACTCCCCTCACACCGAGGAA-3'

PPV-CP (~70 bp)

Forward: 5'-GCAGGCAAGCCGATTGTAGT-3'

Reverse: 5'-TGTATGACTGGAGGTGGTTGAAGT-3'

Deficiencies: None.

DATA EVALUATION RECORD

Review by: Joel V. Gagliardi, Ph.D.
Secondary Review by: John L. Kough, Ph.D.

Study Type	Waiver request for: Acute Oral Toxicity/Pathogenicity (OPPTS 152-30 / 885.3050); Acute Toxicology, Tier II (OPPTS 885.3550); Subchronic Toxicity/Pathogenicity (OPPTS 885.3600); Chronic Feeding Study, Tier III (OPPTS 152-50).
MRID No.	471573-03; 494749-02.
Test Material	Plum Pox Viral Coat Protein.
Study Nos.	IR-4 PR No. 0377B.
Sponsor	Ralph Scorza, Ph.D.; USDA-ARS, Appalachian Fruit Research Station; Kearneysville, West Virginia.
Testing Facility	USDA-ARS, Appalachian Fruit Research Station; Kearneysville, West Virginia.
Titles of Reports	C5 HoneySweet Plum Resistant to Plum Pox Virus (Plum Pox Viral Coat Protein Gene); Amendment Number 1 to MRID 4715730-03.
Authors	Ralph Scorza, Ph.D.; Michael Braverman, Ph.D.
Studies Completed	June 7, 2007; July 10, 2008.
Study Summary	There are several pertinent issues for discussion concerning potential for dietary hazards from C5 Honeysweet Plum. The first is whether there are any novel exposures to proteins that may be toxic, allergenic or that may have antinutrient qualities. Sufficient information has been reviewed in general that indicates that naturally occurring proteins of plant viruses, including the coat protein from Plum Pox Potyvirus, do not possess any qualities that may lead to toxicity, allergenicity or action as an antinutrient when ingested. Bolstering this assessment is a recent study that looked at RNA virus prevalence in the human gastrointestinal tract and found "[t]he vast majority of the 36,769 viral sequences obtained were similar to plant pathogenic RNA viruses. The most abundant fecal virus in this study was pepper mild mottle virus (PMMV), which was found in high concentrations—up to 10^9 virions per gram of dry weight fecal matter". Another key point is the Potyviridae contain one or more polyproteins that are produced directly from virus mRNA, which then self-cleaves into individual proteins, including the coat protein. Exposure in foods from a natural plant virus infection therefore is to a wider range of proteins than just the coat protein. Also pertinent is the mode of resistance elicited by transcription of DNA antisense to the mRNA of the Plum Pox Virus coat protein. Post-transcriptional gene silencing virtually eliminates the possibility of translation of protein from mRNA since homologous mRNA (plant- or virus-expressed) is quickly cleaved. Therefore, exposure to any proteins from the Plum Pox Virus will be virtually eliminated since the mRNA from infecting virus would not be able to replicate under foreseeable agronomic circumstances. Also pertinent to this discussion is the product of the plum pox virus coat protein gene as inserted into the C5 Honeysweet Plum. In the original virus and its replication intermediates, DNA is not required since a virus-encoded RNA-Dependent RNA-polymerase is used. To express the gene in a plant, a DNA copy must be incorporated so that the plant will express mRNA homologous to the virus coat protein only. Often for a Potyvirus this means also adding a start codon and short leader sequence since the viral start codon is distant from the coat protein sequence in the normal viral RNA genome. As mentioned in this petition a full sequence analysis and database search was undertaken including the putative protein sequence of the Plum Pox Virus coat protein gene inserted into C5 Honeysweet Plum. These results (reviewed in the next sections) indicate no similarity to known toxins, allergens or antinutrients. Considering potential production of a protein even when no measurable protein is found is important since the silenced inserted gene has the capability to produce a protein (has an open reading frame) and in some cases PTGS can be suppressed. Known instances where suppression of gene-silencing can occur include low temperature growth and production of PTGS inhibitors. There are however, no foreseeable events that would cause a breakdown in resistance for C5 HoneySweet plum to Plum Pox Virus in the field.
Classification	**ACCEPTABLE.**
Good Laboratory Practice	This research was not conducted in compliance with 40CFR Part 160, but is considered scientifically valid.

I. WAIVER JUSTIFICATION:

The registrant is requesting data waivers for oral toxicity and pathogenicity testing based on the following:

1. There is a long history of mammalian consumption of the entire plant virus particles in food, without causing any known deleterious human health effects or any evidence of toxicity.
2. Virus infected plants are part of the human diet and there have been no findings which indicate that plant viruses are able to replicate in mammals or other vertebrates, thereby eliminating the possibility of human infection.
3. The portion of the genome coding for resistance to plum pox (viral coat protein) and components of the resistance gene as expressed in the plant are incapable of forming infectious particles.
4. Non-occupational exposure such as drinking water exposure is minimal to non-existent since the gene is only expressed within plant tissues.
5. The C5 plum pox resistant plum does not represent a source of novel potential allergenic or antinutrient proteins. A study on the potential allergenicity homology is reviewed below.
6. It is highly unlikely that any protein will be expressed based on the PTGS mode of action and submitted field expression data.

II. PREVIOUS SCIENTIFIC FINDINGS:

EPA has recently reviewed the safety of exposure for plant expression of plant virus components, namely coat proteins (Refs. 1, 2, 3). "EPA's base of experience with viruses infecting food plants has led the Agency to draw three conclusions on which it would rely to support any tolerance exemption for residues of PVC-proteins in food. First, virus-infected plants have always been a part of the human and domestic animal food supply. Most crops are frequently infected with plant viruses, and food from these crops has been and is being consumed without adverse human or animal health effects. Second, plant viruses are not infectious to humans, including children and infants, or to other mammals. Third, plant virus coat proteins, while widespread in food have not been associated with toxic effects to animals or humans."

1. *Always been part of food supply without adverse effects*

Virus-infected food plants have always been a part of the human and domestic animal food supply (Refs. 4, 5, 6, 7, 8, 9). Most plants are infected by at least one virus, and components of plant viruses, including coat proteins, are often found in the produce of crop plants. For example, at the beginning of this century virtually every commercial cultivar of potatoes grown in the United States and Europe was infected with either one or a complex of potato viruses (Ref. 9). Even plants that show no disease symptoms are often found to be infected with viruses (Refs. 8, 10). In addition, a common agricultural practice used since the 1920s for protection against viruses involves intentionally inoculating healthy plants with a mild form of a virus in order to prevent infection by a more virulent form (Ref. 10). A great deal of information supports the ubiquitous appearance of plant viruses in foods, and to date there have been no reports of adverse human or animal health effects associated with consumption of plant viruses in food.

The National Research Council (NRC) observed in its 2000 report that "[h]uman or animal consumption of plants with viral coat proteins is widely considered to be safe, on the basis of common exposure to these types of proteins in nontransgenic types of food" (Ref. 11). The FIFRA SAP at its December 18, 1992 meeting (Ref. 12), also addressed the issue of dietary risk. The SAP stated that "[s]ince viruses are ubiquitous in the agricultural environment at levels higher than will be present in transgenic plants, and there has been a long history of 'contamination' of the food supply by virus coat protein, there is scientific rationale for exempting transgenic plants expressing virus coat protein from the requirement of a tolerance." The FIFRA SAP again discussed PVC-

proteins on October 11-13, 2004 and "agreed that (because of the human history of consuming virus infected food), unaltered PVCPs do not present new dietary exposures" (Ref. 13).
In general, EPA anticipates that dietary exposure through human and animal consumption of plants containing residues of PVC-proteins [.....] will be similar to or less than the dietary exposure to plant virus coat proteins currently found in food plants naturally infected with viruses. Experiments have shown that PVC-protein levels in plants resistant to a virus because of a PVCP-PIP, even when the resistance is mediated by the PVC-protein itself, can be up to one hundred- to one thousand-fold lower in concentration than the level of coat protein found in plants naturally infected by viruses (Refs. 7, 14). The difference in amount of PVC-protein present is even more marked for virus-resistant plants employing resistance mediated by RNA. In such cases, little to no detectable coat protein is produced in a plant containing a PVCP-PIP (Refs. 3, 15). Such information conforms to information EPA has received from the scientific advisory groups the Agency has consulted.

2. *Not infectious to humans*
Any virus/host relationship is characterized by a high degree of specificity (Ref. 7). Plant viruses usually infect plants only within a certain taxonomic group and are unable to infect humans or other vertebrates (Refs. 16, 17). Cellular machinery for [virus attachment and] processing genetic material is highly specific. For example, plant viruses are unable to recognize and attach to the specific sites on mammalian cells needed to penetrate the cell membrane, and plant viruses cannot be processed by mammalian cellular machinery. Plant viruses therefore do not and cannot infect mammals and other vertebrates. In addition, multiple virus components in addition to the coat protein have a role in and are necessary for plant infection. Plant viral coat proteins alone are not infectious to plants, and whole, intact plant viruses are not infectious to humans. Therefore, it is reasonable to assume that a single component of plant viruses, e.g., the PVC-protein, will not be infectious to humans.

3. *No toxic effects to animals or humans*
Humans and domestic animals have been and are exposed to plant viruses in the food supply because most crops are frequently infected with plant viruses. Food from these crops has been and is being consumed without human or animal toxicity related to plant virus infections. Additional evidence of a lack of toxicity can be deduced from the common practice of injecting laboratory animals with purified plant virus preparations [to generate antibodies] without any adverse effects on the animals (Ref. 15). Furthermore, the Agency is not aware of any coat protein from a virus that naturally infects plants that has been identified as a food allergen for humans. Finally, the amount of PVC-protein likely to be found in food is anticipated to be lower than the amount of virus coat protein found in food naturally infected with plant viruses (as discussed in Unit II.A.1).

1. Draft Approach to Exempting Certain PVCP-PIPs from Regulation under FIFRA. Presented to the EPA Science Advisory Panel December 6-7, 2005.
2. Draft Approach to Exempting Certain PVC-Proteins from the Requirement of a Tolerance under FFDCA. Presented to the EPA Science Advisory Panel December 6-7, 2005.
3. Environmental Risk Assessment of Plant Incorporated Protectant (PIP) Inert Ingredients. Presented to the EPA Science Advisory Panel December 6-7, 2005.
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8. Jones, L, E. Anderson, G. Burnett. 1934. The latent virus of potatoes. *Journal of Phytopathology* 7:93-115.
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12. U.S. Environmental Protection Agency. Minutes of the December 18, 1992 FIFRA Scientific Advisory Panel (Subpanel on Plant Pesticides) Meeting on A Set of Scientific Issues Being Considered by the Agency in Connection with the Proposed Regulation of Plant Pesticides.
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15. Hull, R. 2002. Matthews' Plant Virology, Fourth ed. San Diego: Academic Press.
16. Miller, J. 2000. Biotech boosts natural bounty. *Today's Chemist at Work* 9:38-44.
17. Elbehri, A. 2005. Biopharming and the Food System: Examining the Potential Benefits and Risks. *AgBioForum* 8:18-25.

III. DISCUSSION:

There are several pertinent issues concerning potential for dietary hazards from C5 Honeysweet Plum. The first is whether there are any novel exposures to proteins that may be toxic, allergenic or that may have antinutrient qualities. Sufficient information indicates that naturally occurring proteins of plant viruses, including the coat protein from Plum Pox Potyvirus, do not possess any qualities that may lead to toxicity, allergenicity or action as an antinutrient when ingested. Bolstering this assessment is a recent study that looked at RNA virus prevalence in the human gastrointestinal tract and found "[t]he vast majority of the 36,769 viral sequences obtained were similar to plant pathogenic RNA viruses. The most abundant fecal virus in this study was pepper mild mottle virus (PMMV), which was found in high concentrations up to 10^9 virions per gram of dry weight fecal matter" (Ref. 19).

Another key point is the Potyviridae contain one or more polyproteins that are produced directly from virus mRNA, which then self-cleaves into individual proteins, including the coat protein (Ref. 18). Exposure in foods from a natural plant virus infection therefore is to a wider range of proteins than just the coat protein. Also pertinent is the mode of resistance elicited by transcription of DNA antisense to the mRNA of the Plum Pox Virus coat protein. Post-transcriptional gene silencing virtually eliminates the possibility of translation of protein from mRNA since homologous mRNA (plant- or virus-expressed) is quickly cleaved and the chromosomal DNA gene is methylated to prevent further transcription. Therefore, exposure to any proteins from the Plum Pox Virus will be virtually eliminated since the mRNA from infecting virus would not be able to replicate under foreseeable agronomic circumstances. Also pertinent to this discussion is the product of the plum pox virus coat protein gene as inserted into the C5 Honeysweet Plum. In the original virus and its replication intermediates, DNA is not required since a virus-encoded RNA-Dependent RNA-polymerase is used. To express the gene in a plant, a DNA copy must be incorporated so that the plant will express mRNA homologous to the virus coat protein only. Often for a Potyvirus this means also adding a start codon and short leader sequence since the start codon for the virus

polyprotein is distant from the coat protein sequence in the normal viral RNA genome. As mentioned in this petition a full sequence analysis and database search was undertaken including the putative protein sequence for the Plum Pox Virus coat protein gene inserted into C5 Honeysweet Plum. These results (reviewed in the next sections) indicate no similarity to known toxins, allergens or antinutrients. Considering potential production of a protein even when no measurable protein is detected is important since the silenced inserted gene has the capability to produce a protein (has an open reading frame) and in some cases PTGS can be suppressed. Known instances where suppression of gene-silencing can occur include low temperature growth (Ref. 20) and production of PTGS inhibitors (Ref. 21). There are however, no foreseeable events that would cause a breakdown in resistance for C5 HoneySweet plum to Plum Pox Virus in the field.

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IV. CONCLUSION: ACCEPTABLE.

DATA EVALUATION RECORD

Review by: Joel V. Gagliardi, Ph.D.
Secondary Review by: John L. Kough, Ph.D.

Study Type	Allergenicity-Homology Study; Report of Hypersensitivity Incidents; In-Vitro Digestibility Study Waiver.
MRID No.	471573-03; 474749-02; 474749-03.
Test Material	Transgene and overlapping sequences of C5 HoneySweet Plum.
Study Nos.	IR-4 PR No. 0377B.
Sponsor	Ralph Scorza, Ph.D.; USDA-ARS, Appalachian Fruit Research Station; Kearneysville, West Virginia.
Testing Facility	USDA-ARS, Appalachian Fruit Research Station; Kearneysville, West Virginia.
Titles of Reports	C5 HoneySweet Plum Resistant to Plum Pox Virus (Plum Pox Viral Coat Protein Gene); Amendment Number 1 to MRID 471573-03.
Authors	Ralph Scorza, Ph.D.; Michael Braverman, Ph.D.
Studies Completed	June 7, 2007; July 10, 2008.
Study Summary	Clones of the inserts (or the putative coat protein) were chosen for analysis due to the possibility for sequence overlap and protein production other than from transgene open reading frames, and the need to assess the actual insert not the intended clone. Open-reading frames analysis was used to compare any alignments of potential allergens and antinutrients to further narrow down any potential hazard. None of the sequence analyses produced alignments of >35% and in the few cases where a direct amino acid match was found, they were common sequences and below the hazard threshold of an 8 amino acid homology currently utilized as a benchmark. Therefore, no hazard potential for allergenic or antinutrient capability apparently exists from the transgenes and overlapping plum DNA expression in the C5 HoneySweet plum. No further testing, including an in-vitro digestibility assay, is required. The registrant also included a statement that since experimentation began with C5 HoneySweet plum in 1992 approximately 80 trees have been tested. ARS production staff numbering approximately 20 people in the U.S. (West Virginia) and personnel performing testing in Spain, Poland, Romania, the Czech Republic and Chile, have not experienced known hypersensitive or other adverse effects.
Classification	**ACCEPTABLE.**
Good Laboratory Practice	This research was not conducted in compliance with 40CFR Part 160, but is considered scientifically valid.

I. MATERIALS AND METHODS:

A. Database Sequence Searches:

Clones of DNA inserts, containing approximately 80% of the combined Plum Pox Virus coat protein sequence and other associated C5 inserts were chosen for analysis. Translation (putative amino acid sequence) was deduced using the EMBOSS transeq utility (Rice 2000) in the direction of the open reading frames. A combination of protein functional analysis and sequence homology searches were utilized to determine similarity to known allergens. The functional analysis employed the European Bioinformatic Institutes (EBI) InterProScan algorithm (Zbobnov 2001), and except for TMHMM and GENE3D, used default settings, and an E-value $\leq$ 0.001 reported as significant.
Following this, the Allergen Database for Food Safety (ADFS) was used to query the same sequences using FAO/WHO 'Consultations of Allergenicity' (Hileman et al. 2002), briefly; FASTA alignments to 80 amino acid sequences screening for a 35% identity to known allergens followed by a $\geq$ 6-8 amino acid 'exact match' search.
Under these constraints a positive result has both a 35% sequence homology (80 amino acid window) and at least one $\geq$ 6-8 amino acid match on the same sequence.

Further testing involved a 'motif similarity analysis' for potentially allergenic similarity (Stadler 2003).
In a response to a deficiency letter the registrant also submitted protein sequence database searches, as above, of just the Plum Pox Virus coat protein sequence from the C5 transformation event, if it were to be produced.
Any matches for potential allergens were then reverse transcribed to match to the corresponding part of the cDNA and mapped to open reading frames or other portions of the sequences tested.

B. Antinutrient Analysis:
Sequence alignment algorithms CLUSTALW (Pillai 2005) and MUSCLE (Edgar 2004) within the NCBI protein database were used to search the keyword 'anti-nutrient'. Transgene sequences were compared with 466 antinutrient sequences compiled from the NCBI database.

Allergen Database for Food Safety. http://allergen.nihs.go.jp/ADFS
Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32(5):1792-1797.
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Pillai, S., V. Silventoinen, K. Kallio, M. Senger, S. Sobhany, J. Tate, S. Velankar, A. Golovin, K. Henrich, P. Rice, P. Stoehr and R. Lopez. 2005. SOAP-based services provided by the European Bioinformative Institute. Nucleic Acids Research 33: W25-W28.
Rice, P., I. Longden and A. Bleasby. 2000. EMBOSS: The European Molecular Biology Open Software Suite. Trends in Genetics 16(6): 276-277.
Stadler, M.B. and B.D. Stadler. 2003. Allergenicity prediction by protein sequence. The FASEB Journal 17:1141-1143.
Zbobnov, E.M. and R. Apweiler. 2001. InterProScan – and integration platform for the signature-recognition methods in InterPro. Bioinformatics Applications Note 17(9):847-848.

II. RESULTS:

No significant alignments to known proteins were returned from a BLASTp ADFS and motif-prediction analysis in all frames. Positive results were returned from these sequences with >35% identity, though concurrent exact matches of $\geq$ 6-8 amino acids to allergens in the databases were less frequent. In fact there were only three hits, one of 6 amino acids to 'PPPPPP' and the others with a 7 amino acid match to 'SSSSSLL'. In all cases the matching amino acid sequence homology was outside the open reading frames of any transgenes. Reported functional similarities using InterProScan alignments did not coincide with any of the regions where allergenic protein sequence alignments results predicted, above. There were no functional similarity regions with the E-value threshold < 0.01 (1 in 100 chances of being significant).

Simple alignment similarity threshold values ranged from E-values of < 0.00008 ($< 8e^{-5}$) for a carbohydrate metabolism enzyme, to scores much lower, in the range of $2.1e^{-257}$. Among the most significant results were for Beta-glucuronidase ($2.1e^{-257}$) and Potyvirus coat protein ($3.4e^{-190}$); genes that were intentionally inserted. Also among significant results ($3.1e^{-225}$) was a protein listed

as "family not named" and with no known associated molecular function or biological process.

Other protein sequences with matches were well-known, such as a carbohydrate metabolism enzyme, glycoside hydrolase family 2 with separate domain matches at $4.7e^{-181}$ and $2.6e^{-68}$. Antibiotic resistance associated sequence alignments included bleomycin resistance protein ($2.5e^{-7}$), aminoglycoside phosphotrasnferase ($1.5e^{-54}$) and beta-lactamase ($8.4e^{-49}$) which are likely similar to inserted gene sequences for neomycin phosphotrasnferase II and the cos-interrupted beta-lactamase in this construct. Using the least significant value for a known inserted gene match, to beta-lactamase ($8.4e^{-49}$) as a cutoff, there were only a few other significant matches; two listed as 'domain specific binding' ($8.8e^{-88}$ and $1e^{-67}$) which are commonly repeated sequences, and a translation regulating protein from Caulimovirus viroplasmin ($2e^{-78}$). Also noted were two matches from different inserts that were below the chosen cutoff, to DNA/RNA polymerases (both $1.1e^{-44}$), likely aligned to parts of the inserts originating from virus sequences.

Sequence database searches of the Plum Pox Virus coat protein sequence (if it were to be produced) yielded only one 6-amino acid match (LNGLMV), in the Coat Protein open reading frame, when compared to the allergen protein database. There is no indication that this short sequence could bind to an immunoglobulin and elicit any immune response.

There were no significant pairwise alignments between transgene sequences and antinutrients from the NCBI protein database. All alignments scored less than a 13% homology.

III. <u>DISCUSSION</u>:

Clones of the inserts (or the putative coat protein) were chosen for analysis due to the possibility for sequence overlap and protein production other than from transgene open reading frames, and the need to assess the actual insert not the intended clone. Open-reading frames analysis was used to compare any alignments of potential allergens and antinutrients to further narrow down any potential hazard. None of the sequence analyses produced alignments of >35% and in the few cases where a direct amino acid match was found, they were common sequences and below the hazard threshold of an 8 amino acid homology currently utilized as a benchmark and indication of ability to bind an immunoglobulin. Therefore, no hazard potential for allergenic or antinutrient capability apparently exists from the transgenes and overlapping plum DNA expression in the C5 HoneySweet plum. No further testing, including an in-vitro digestibility assay, is required.

The registrant also included a statement that since experimentation began with C5 HoneySweet plum in 1992 approximately 80 trees have been tested. ARS production staff numbering approximately 20 people in the U.S. (West Virginia) and personnel performing testing in Spain, Poland, Romania, the Czech Republic and Chile, have not experienced known hypersensitive or other adverse effects.

IV. <u>CONCLUSION</u>: ACCEPTABLE.

DATA EVALUATION RECORD	
EPA Review by: Joel V. Gagliardi, Ph.D.	
EPA Secondary Review by: John L. Kough, Ph.D.	
Study Type	Tolerance Exemption Petition.
MRID No.	Not assigned.
Test Material	C5 HoneySweet Plum – Resistant to Plum Pox Virus (Plum Pox Viral Coat Protein Gene).
Study Nos.	IR-4 PR No. 0377B.
Sponsor	Ralph Scorza, Ph.D.; USDA-ARS, Appalachian Fruit Research Station; Kearneysville, West Virginia.
Testing Facility	USDA-ARS, Appalachian Fruit Research Station; Kearneysville, West Virginia.
Titles of Reports	Tolerance Petition: Establishment of an Exemption from Tolerance for Plum Pox Viral Coat Protein in or on Fruit, Stone, Group 12 and Almond.
Authors	Michael Braverman, Ph.D.
Studies Completed	June 11, 2007; Amended September 28, 2007.
Study Summary	The submitted data and gathered background information on the safety of plant virus proteins, including coat proteins in foods and namely the C5 clone that can express the Plum Pox Virus coat protein with short leader sequence from C5 HoneySweet Plum, led to the conclusion that the C5 Plum Pox Virus Resistance is low risk and safe for consumption in foods. Due to outcrossing potential and the various uses of rootstocks and grafts, a wider tolerance exemption petition encompassing all stone fruits was reviewed. The safe for consumption and low risk finding holds equally for all foods in this group. In addition to a long history of consumption without known toxic, allergenic or antinutrient effects for all proteins from plant viruses, the C5 specific sequence and putative coat protein representing genome sequencing comprising ~80% of the transgenes was used to search databases for any match. No qualifying toxic, allergenic or antinutrient sequence alignments were found. In the specific case of C5 Honeysweet Plum the mode of resistance is post-transcriptional-gene-silencing, which renders the C5 genome incapable of producing mRNA to express protein under normal agronomic circumstances. No PPV coat protein has been detected to date in these plum trees or fruits. In addition, infecting Plum Pox Virus is prevented from expressing symptoms and in many cases infection is prevented, thereby lowering exposure to protein even from the naturally occurring virus. While the safe for consumption and low-risk findings here were not based on the premise that exposure cannot occur, actual exposure to Plum Pox Virus proteins and to the cloned PPV coat protein is expected to be much lower (possibly non-existent) compared to PPV-infected trees and fruits.
Classification	**ACCEPTABLE** – C5 Plum Pox Viral Coat Protein in or on Fruit, Stone, Group 12 and Almond.

BACKGROUND:

A. Rationale and discussion for oral exposure waiver requests:

I. WAIVER JUSTIFICATION:

The registrant is requesting data waivers for oral toxicity and pathogenicity testing based on the following:

1. There is a long history of mammalian consumption of entire plant virus particles in food, without causing any known deleterious human health effects or any evidence of toxicity.
2. Virus infected plants are part of the human diet and there have been no findings which indicate that plant viruses are able to replicate in mammals or other vertebrates, thereby eliminating the possibility of human infection.
3. The portion of the genome coding for resistance to plum pox (viral coat protein) and subcomponents of the resistance gene expressed in the plant are incapable of forming infectious particles.
4. Non-occupational exposure such as drinking water exposure is minimal to non-existent since

the gene is only expressed within plant tissues.
5. The C5 plum pox resistant plum does not represent a source of novel potential allergenic or antinutrient proteins. A study on the potential allergenicity homology is reviewed herein.
6. It is highly unlikely that any protein will be expressed based on the PTGS mode of action and field expression data.

II. PREVIOUS SCIENTIFIC FINDINGS:

EPA has recently reviewed the safety of exposure for plant expression of plant virus components, namely coat proteins (Refs. 1, 2, 3). "EPA's base of experience with viruses infecting food plants has led the Agency to draw three conclusions on which it would rely to support any tolerance exemption for residues of PVC-proteins in food. First, virus-infected plants have always been a part of the human and domestic animal food supply. Most crops are frequently infected with plant viruses, and food from these crops has been and is being consumed without adverse human or animal health effects. Second, plant viruses are not infectious to humans, including children and infants, or to other mammals. Third, plant virus coat proteins, while widespread in food have not been associated with toxic effects to animals or humans."

1. *Always been part of food supply without adverse effects*

Virus-infected food plants have always been a part of the human and domestic animal food supply (Refs. 4, 5, 6, 7, 8, 9). Most plants are infected by at least one virus, and components of plant viruses, including coat proteins, are often found in the produce of crop plants. For example, at the beginning of this century virtually every commercial cultivar of potatoes grown in the United States and Europe was infected with either one or a complex of potato viruses (Ref. 9). Even plants that show no disease symptoms are often found to be infected with viruses (Refs. 8, 10). In addition, a common agricultural practice used since the 1920s for protection against viruses involves intentionally inoculating healthy plants with a mild form of a virus in order to prevent infection by a more virulent form (Ref. 10). A great deal of information supports the ubiquitous appearance of plant viruses in foods, and to date there have been no reports of adverse human or animal health effects associated with consumption of plant viruses in food.

The National Research Council (NRC) observed in its 2000 report that "[h]uman or animal consumption of plants with viral coat proteins is widely considered to be safe, on the basis of common exposure to these types of proteins in nontransgenic types of food" (Ref. 11). The FIFRA SAP at its December 18, 1992 meeting (Ref. 12), also addressed the issue of dietary risk. The SAP stated that "[s]ince viruses are ubiquitous in the agricultural environment at levels higher than will be present in transgenic plants, and there has been a long history of 'contamination' of the food supply by virus coat protein, there is scientific rationale for exempting transgenic plants expressing virus coat protein from the requirement of a tolerance." The FIFRA SAP again discussed PVC-proteins on October 11-13, 2004 and "agreed that (because of the human history of consuming virus infected food), unaltered PVCPs do not present new dietary exposures" (Ref. 13).

In general, EPA anticipates that dietary exposure through human and animal consumption of plants containing residues of PVC-proteins [.....] will be similar to or less than the dietary exposure to plant virus coat proteins currently found in food plants naturally infected with viruses. Experiments have shown that PVC-protein levels in plants resistant to a virus because of a PVCP-PIP, even when the resistance is mediated by the PVC-protein itself, can be up to one hundred- to one thousand-fold lower in concentration than the level of coat protein found in plants naturally infected by viruses (Refs. 7, 14). The difference in amount of PVC-protein present is even more marked for virus-resistant plants employing resistance mediated by RNA. In such cases, little to no detectable coat protein is produced in a plant containing a PVCP-PIP

(Refs. 3, 15). Such information conforms to information EPA has received from the scientific advisory groups the Agency has consulted.

2. *Not infectious to humans*
Any virus/host relationship is characterized by a high degree of specificity (Ref. 7). Plant viruses usually infect plants only within a certain taxonomic group and are unable to infect humans or other vertebrates (Refs. 16, 17). Cellular machinery for [virus attachment and] processing genetic material is highly specific. For example, plant viruses are unable to recognize and attach to the specific sites on mammalian cells needed to penetrate the cell membrane, and plant viruses cannot be processed by mammalian cellular machinery. Plant viruses therefore do not and cannot infect mammals and other vertebrates. In addition, multiple virus components in addition to the coat protein have a role in and are necessary for plant infection. Plant viral coat proteins alone are not infectious to plants, and whole, intact plant viruses are not infectious to humans. Therefore, it is reasonable to assume that a single component of plant viruses, e.g., the PVC-protein, will not be infectious to humans.

3. *No toxic effects to animals or humans*
Humans and domestic animals have been and are exposed to plant viruses in the food supply because most crops are frequently infected with plant viruses. Food from these crops has been and is being consumed without human or animal toxicity related to plant virus infections. Additional evidence of a lack of toxicity can be deduced from the common practice of injecting laboratory animals with purified plant virus preparations [to generate antibodies] without any adverse effects on the animals (Ref. 15). Furthermore, the Agency is not aware of any coat protein from a virus that naturally infects plants that has been identified as a food allergen for humans. Finally, the amount of PVC-protein likely to be found in food is anticipated to be lower than the amount of virus coat protein found in food naturally infected with plant viruses (as discussed in Unit II.A.1).

1. Draft Approach to Exempting Certain PVCP-PIPs from Regulation under FIFRA. Presented to the EPA Science Advisory Panel December 6-7, 2005.
2. Draft Approach to Exempting Certain PVC-Proteins from the Requirement of a Tolerance under FFDCA. Presented to the EPA Science Advisory Panel December 6-7, 2005.
3. Environmental Risk Assessment of Plant Incorporated Protectant (PIP) Inert Ingredients. Presented to the EPA Science Advisory Panel December 6-7, 2005.
4. Dewan, C., M.N. Pearson. 1995. Natural field infection of garlic by garlic yellow streak virus in the Pukekohe area of New Zealand and associated problems with the introduction of new garlic cultivars. *New Zealand Journal of Crop and Horticultural Science* 23:97-102.
5. McKinney, H.H. 1929. Mosaic diseases in the Canary Islands, West Africa, and Gibraltar. *Journal of Agricultural Research* 39:557-78.
6. Provvidenti, R, D. Gonsalves. 1984. Occurrence of zucchini yellow mosaic virus in cucurbits from Connecticut, New York, Florida, and California. *Plant Disease* 68:443-6.
7. Palukaitis, P. 1991. Virus-mediated genetic transfer in plants. In: Levin, M, Strauss, H. Risk Assessment in Genetic Engineering. New York: McGraw-Hill, pp. 140-62.
8. Jones, L, E. Anderson, G. Burnett. 1934. The latent virus of potatoes. *Journal of Phytopathology* 7:93-115.
9. Beemster, A.B.R., J.A. de Bokx. 1987. Survey of properties and symptoms. *In*: J.A. de Bokx AND J.P.H. van der Want *eds*. Viruses of Potatoes and Seed Potato Production. Wageningen: Pudoc, pp. 84-93.
10. Fulton, R. 1986. Practices and precautions in the use of cross protection for plant virus disease control. *Annual Review of Phytopathology* 24:67-81.
11. National Research Council. 2000. Genetically Modified Pest-Protected Plants: Science and Regulation. Washington, DC: National Academy Press.

12. U.S. Environmental Protection Agency. Minutes of the December 18, 1992 FIFRA Scientific Advisory Panel (Subpanel on Plant Pesticides) Meeting on A Set of Scientific Issues Being Considered by the Agency in Connection with the Proposed Regulation of Plant Pesticides.
13. U.S. Environmental Protection Agency. Minutes of the October 13-15, 2004 FIFRA Scientific Advisory Panel Meeting on Issues Associated with Deployment of a Type of Plant-Incorporated Protectant (PIP), Specifically those Based on Plant Viral Coat Proteins (PVCP-PIPs). 2004.
14. Quemada, H. 1994. Food safety evaluation of a transgenic squash. OECD Workshop on Food: Provisionsal Proceedings of the Safety Evaluation. Paris: OECD, pp. 71-9.
15. Hull, R. 2002. Matthews' Plant Virology, Fourth ed. San Diego: Academic Press.
16. Miller, J. 2000. Biotech boosts natural bounty. *Today's Chemist at Work* 9:38-44.
17. Elbehri, A. 2005. Biopharming and the Food System: Examining the Potential Benefits and Risks. *AgBioForum* 8:18-25.

III. DISCUSSION:

There are several pertinent issues for discussion concerning potential for dietary hazards from C5 Honeysweet Plum. The first is whether there are any novel exposures to proteins that may be toxic, allergenic or that may have antinutrient qualities. Sufficient information has been reviewed in general that indicates that naturally occurring proteins of plant viruses, including the coat protein from Plum Pox Potyvirus, do not possess any qualities that may lead to toxicity, allergenicity or action as an antinutrient when ingested. Bolstering this assessment is a recent study that looked at RNA virus prevalence in the human gastrointestinal tract and found "[t]he vast majority of the 36,769 viral sequences obtained were similar to plant pathogenic RNA viruses. The most abundant fecal virus in this study was pepper mild mottle virus (PMMV), which was found in high concentrations—up to 10^9 virions per gram of dry weight fecal matter" (Ref. 19).
Another key point is the Potyviridae contain one or more polyproteins that are produced directly from virus mRNA, which then self-cleaves into individual proteins, including the coat protein (Ref. 18). Exposure in foods from a natural plant virus infection therefore is to a wider range of proteins than just the coat protein. Also pertinent is the mode of resistance elicited by transcription of DNA antisense to the mRNA of the Plum Pox Virus coat protein. Post-transcriptional gene silencing virtually eliminates the possibility of translation of protein from mRNA since homologous mRNA (plant- or virus-expressed) is quickly cleaved and the chromosomal DNA gene is methylated to prevent further transcription.

Therefore, exposure to any proteins from the Plum Pox Virus will be virtually eliminated since the mRNA from infecting virus would not be able to replicate under foreseeable agronomic circumstances. Also pertinent to this discussion is the product of the plum pox virus coat protein gene as inserted into the C5 Honeysweet Plum. In the original virus and its replication intermediates, DNA is not required since a virus-encoded RNA-Dependent RNA-polymerase is used. To express the gene in a plant, a DNA copy must be incorporated so that the plant will express mRNA homologous to the virus coat protein only. Often for a Potyvirus this means also adding a start codon and short leader sequence since the start codon for the virus polyprotein is distant from the coat protein sequence in the normal viral RNA genome. As mentioned in this petition a sequence analysis and database search was undertaken including the putative protein sequence for the Plum Pox Virus coat protein gene inserted into C5 Honeysweet Plum. These results indicate no similarity to known toxins, allergens or antinutrients. Considering potential production of a protein even when no measurable protein is detected is important since the silenced inserted gene has the capability to produce a protein (has an open reading frame) and in some cases PTGS can be suppressed. Known instances where suppression of gene-silencing can occur include low temperature growth (Ref. 20) and production of PTGS inhibitors (Ref. 21). There are however,

no foreseeable events that would cause a breakdown in resistance for C5 HoneySweet plum to Plum Pox Virus in the field.

18. Shukla, D.D., C.W. Ward. A.A. Brunt. 1994. The Potyviridae. CAB International. University Press. Cambridge, UK.
19. Zhang, T., M. Breitbart, W.H. Lee1, J.-Q. Run, C.L. Wei, S.W.L. Soh, M.L. Hibberd, E.T. Liu, F. Rohwer, Y. Ruan. 2006. RNA Viral Community in Human Feces: Prevalence of Plant Pathogenic Viruses. PLOS Biology 4(1):108-118.
20. Gyorgy, S., D. Silhavy, A. Molnar, Z. Havelda, A. Lovas, L. Lakatos, Z. Banfalvi and J. Burgyan. 2003. Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. The EMBO Journal 22(3):633-640.
21. Guillaume, M. and O. Voinnet. 2004. Viral suppression of RNA silencing in plants. Molecular Plant Pathology 5(1):71.82.

B. Rationale for Allergenicity-Homology Study; Report of Hypersensitivity Incidents; In-Vitro Digestibility Study Waiver:

A. **Database Sequence Searches:**

Clones of DNA inserts, containing approximately 80% of the combined Plum Pox Virus coat protein sequence and other associated C5 inserts were chosen for analysis. Translation (putative amino acid sequence) was deduced using the EMBOSS transeq utility (Rice 2000) in the direction of the open reading frames. A combination of protein functional analysis and sequence homology searches were utilized to determine similarity to known allergens. The functional analysis employed the European Bioinformatic Institutes (EBI) InterProScan algorithm (Zbobnov 2001) and except for TMHMM and GENE3D used default settings, and an E-value $\leq$ 0.001 reported as significant.

Following this, the Allergen Database for Food Safety (ADFS) was used to query the same sequences using FAO/WHO 'Consultations of Allergenicity' (Hileman et al. 2002), briefly; FASTA alignments to 80 amino acid sequences screening for a 35% identity to known allergens followed by a $\geq$ 6-8 amino acid 'exact match' search.

Under these constraints a positive result has both a 35% sequence homology (80 amino acid window) and at least one $\geq$ 6-8 amino acid match on the same sequence.

Further testing involved a 'motif similarity analysis' for potentially allergenic similarity (Stadler 2003). In a response to a deficiency letter the registrant also submitted protein sequence database searches, as above, of just the Plum Pox Virus coat protein sequence from the C5 transformation event, if it were to be produced.

Any matches for potential allergens were then reverse transcribed to match to the corresponding part of the cDNA and mapped to open reading frames or other portions of the sequences tested.

B. Antinutrient Analysis:

Sequence alignment algorithms CLUSTALW (Pillai 2005) and MUSCLE (Edgar 2004) within the NCBI protein database were used to search the keyword 'anti-nutrient'. Transgene sequences were compared with 466 antinutrient sequences compiled from the NCBI database.

Allergen Database for Food Safety. http://allergen.nihs.go.jp/ADFS
Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32(5):1792-1797.
FAO/WHO. 2001. Evaluation of Allergenicity of Genetically Modified Foods. Report of a Joint FAO/WHO Expert Consultation of Allergenicity of Foods Derived from Biotechnology, 22-25

January 2001.
Hileman, R.E., A. Silvanovich, R.E. Goodman, E.A. Rice, G. Holleschak, J.D. Astwood and S.L. Hefle. 2002. Bioinformatice Methods for Allergenicity Assessment Using a Comprehensive Allergen Database. Allergy and Immunology 128:280-291.
National Center for Biological Information BLAST databases. http://blast.ncbi.nlm.nih.gov/Blast.cgi
Pillai, S., V. Silventoinen, K. Kallio, M. Senger, S. Sobhany, J. Tate, S. Velankar, A. Golovin, K. Henrich, P. Rice, P. Stoehr and R. Lopez. 2005. SOAP-based services provided by the European Bioinformative Institute. Nucleic Acids Research 33: W25-W28.
Rice, P., I. Longden and A. Bleasby. 2000. EMBOSS: The European Molecular Biology Open Software Suite. Trends in Genetics 16(6): 276-277.
Stadler, M.B. and B.D. Stadler. 2003. Allergenicity prediction by protein sequence. The FASEB Journal 17:1141-1143.
Zbobnov, E.M. and R. Apweiler. 2001. InterProScan – and integration platform for the signature-recognition methods in InterPro. Bioinformatice Applications Note 17(9):847-848.

II. RESULTS:

No significant alignments to known proteins were returned from a BLASTp ADFS and motif-prediction analysis in all frames. Positive results were returned from these sequences with >35% identity, though concurrent exact matches of ≥ 6-8 amino acids to allergens in the databases were less frequent. In fact there were only three hits, one of 6 amino acids to 'PPPPPP' and the others with a 7 amino acid match to 'SSSSSLL'. In all cases the matching amino acid sequence homology was outside the open reading frames of any transgenes. Reported functional similarities using InterProScan alignments did not coincide with any of the regions where allergenic protein sequence alignments results predicted, above. There were no functional similarity regions with the E-value threshold < 0.01 (1 in 100 chances of being significant).

Simple alignment similarity threshold values ranged from E-values of < 0.00008 (< $8e^{-5}$) for a carbohydrate metabolism enzyme, to scores much lower, in the range of $2.1e^{-257}$. Among the most significant results were for Beta-glucuronidase ($2.1e^{-257}$) and Potyvirus coat protein ($3.4e^{-190}$); genes that were intentionally inserted. Also among significant results ($3.1e^{-225}$) was a protein listed as "family not named" and with no known associated molecular function or biological process.

Other protein sequences with matches were well-known, such as a carbohydrate metabolism enzyme, glycoside hydrolase family 2 with separate domain matches at $4.7e^{-181}$ and $2.6e^{-68}$. Antibiotic resistance associated sequence alignments included bleomycin resistance protein ($2.5e^{-7}$), aminoglycoside phosphotrasnferase ($1.5e^{-54}$) and beta-lactamase ($8.4e^{-49}$) which are likely similar to inserted gene sequences for neomycin phosphotrasnferase II and the cos-interrupted beta-lactamase in this construct. Using the least significant value for a known inserted gene match, to beta-lactamase ($8.4e^{-49}$) as a cutoff, there were only a few other significant matches; two listed as 'domain specific binding' ($8.8e^{-88}$ and $1e^{-67}$) which are commonly repeated sequences, and a translation regulating protein from Caulimovirus viroplasmin ($2e^{-78}$). Also noted were two matches from different inserts that were below the chosen cutoff, to DNA/RNA polymerases (both $1.1e^{-44}$), likely aligned to parts of the inserts originating from virus sequences.

Sequence database searches of the Plum Pox Virus coat protein sequence (if it were to be produced) yielded only one 6-amino acid match (LNGLMV), in the Coat Protein open reading frame, when compared to the allergen protein database. There is no indication that this short sequence could bind to an immunoglobulin and elicit any immune response.

There were no significant pairwise alignments between transgene sequences and antinutrients from the NCBI protein database. All alignments scored less than a 13% homology.

III. DISCUSSION:

Clones of the inserts (or the putative coat protein) were chosen for analysis due to the possibility for sequence overlap and protein production other than from transgene open reading frames, and the need to assess the actual insert not the intended clone. Open-reading frames analysis was used to compare any alignments of potential allergens and antinutrients to further narrow down any potential hazard. None of the sequence analyses produced alignments of >35% and in the few cases where a direct amino acid match was found, they were common sequences and below the hazard threshold of an 8 amino acid homology currently utilized as a benchmark. Therefore, no hazard potential for allergenic or antinutrient capability apparently exists from the transgenes and overlapping plum DNA expression in the C5 HoneySweet plum. No further testing, including an in-vitro digestibility assay, is required.

The registrant also included a statement that since experimentation began with C5 HoneySweet plum in 1992 approximately 80 trees have been tested. ARS production staff numbering approximately 20 people in the U.S. (West Virginia) and personnel performing testing in Spain, Poland, Romania, the Czech Republic and Chile, have not experienced known hypersensitive or other adverse effects.

GENERAL DISCUSSION:

The submitted data and gathered background information on the safety of plant virus proteins, including coat proteins in foods and namely the C5 clone that can express the Plum Pox Virus coat protein with short leader sequence from C5 HoneySweet Plum, led to the conclusion that the C5 Plum Pox Virus Resistance is low risk and safe for consumption in foods. Due to outcrossing potential and the various uses of rootstocks and grafts, a wider tolerance exemption petition encompassing all stone fruits was reviewed. The safe for consumption and low risk finding holds equally for all foods in this group. In addition to a long history of consumption without known toxic, allergenic or antinutrient effects for all proteins from plant viruses, the C5 specific sequence and putative coat protein representing genome sequencing comprising ~80% of the transgenes was used to search databases for any match. No qualifying toxic, allergenic or antinutrient sequence alignments were found. In the specific case of C5 Honeysweet Plum the mode of resistance is post-transcriptional-gene-silencing, which renders the C5 genome incapable of producing mRNA to express protein under normal agronomic circumstances. No PPV coat protein has been detected to date in these plum trees or fruits. In addition, infecting Plum Pox Virus is prevented from expressing symptoms and in many cases infection is prevented, thereby lowering exposure to protein even from the naturally occurring virus. While the safe for consumption and low-risk findings here were not based on the premise that exposure cannot occur, actual exposure to Plum Pox Virus proteins and to the cloned PPV coat protein is expected to be much lower (possibly non-existent) compared to the wild-type trees and fruits.

CLASSIFICATION:

ACCEPTABLE – C5 Plum Pox Viral Coat Protein in or on Fruit, Stone, Group 12 and Almond.

13544

R179256

Chemical Name: Plum Pox Resistance Gene (Plum Pox Viral Coat Protein Gene)

PC Code: 006354
HED File Code: 41500 BPPD Tox/Chem
Memo Date: 3/19/2009
File ID: DPD342996
DPD343005
DPD343006
DPD347035
DPD355577
DPD355580
Accession #: 000-00-0132

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